JOURNAL OF VIROLOGY, Jan. 1997, p. 337–344 Vol. 71, No. 1
0022-538X/97/$04.00+0

Function of the Human T-Cell Leukemia Virus Type 1 21-Base-Pair Repeats in Basal Transcription

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Received 27 June 1996/Accepted 23 September 1996

The human T-cell leukemia virus type 1 (HTLV-1) promoter contains three copies of an imperfect 21-bp repeat called Tax-responsive element (TRE1). To examine the role of individual TRE1 sequences in basal transcription of the HTLV-1 promoter, site-directed mutations were generated in all possible combinations of one, two, or all three TRE1 elements in the viral long terminal repeat (LTR) and tested in vivo for transcriptional activity. Mutation of the middle TRE1 resulted in the greatest reduction in basal activity. Electrophoretic mobility shift analysis demonstrated that the protein complexes bound to each of the three TRE1 sequences were not identical. The complexes formed with the TATA-distal and middle TRE1s were dependent on the core cyclic AMP response element (CRE) found in all three TRE1s, while the cellular transcription factor Sp1 bound the TATA-proximal TRE1 in a CRE-independent manner. Sp1 binding produced a footprint on the viral LTR which covered the 5′ region of the proximal TRE1. Mixing experiments demonstrated that the bindings of CREB and Sp1 to the proximal TRE1 were mutually exclusive. Sp1 was able to activate transcription both from the complete LTR and from the proximal TRE1 alone. These studies demonstrate that the TRE1 elements in the HTLV-1 LTR are functionally nonequivalent and suggest that Sp1 can influence HTLV-1 basal transcription.

Human T-cell leukemia virus type 1 (HTLV-1) is the etiologic agent of both adult T-cell leukemia and tropical spastic paraparesis/HTLV-1 associated myelopathy (16, 34). Following infection, the HTLV-1 provirus integrates into the host genome, where it can remain latent for several decades. Basal transcription from the viral promoter produces unspliced and singly spliced mRNAs that encode the Gag, Pol, and Env proteins as well as a doubly spliced mRNA that encodes Tax, Rex, and p21. The regulation of basal transcription is likely to play an important role in viral latency.

The Tax protein is a potent stimulator of viral transcription (5, 12, 38) and is the transforming protein of HTLV-1 (19–21, 30, 35, 41). Tax does not bind DNA directly but rather associates via protein-protein interactions with cellular proteins bound to the viral promoter (7, 15, 27, 46). Because of its critical role in viral replication and pathogenesis, much effort has been devoted to identifying Tax-responsive elements within the viral promoter and the proteins which bind these elements. The viral promoter is located in the 5′ long terminal repeat (LTR) and contains three copies of a 21-bp imperfect repeat called Tax-responsive element 1 (TRE1) (5, 13, 33). Each copy of TRE1 contains a conserved cyclic AMP-responsive element (CRE) core flanked by GC-rich regions (24). Cellular proteins such as HEB, TREB1, TREB2, CRE-binding protein (CREB), and ATF1 have been shown to bind TRE1 in vitro (3, 40, 43, 45). Two of these proteins, CREB and ATF1, have also been shown to interact with Tax in vitro (47). The ability of proteins that bind to TRE1 to regulate transcription of cellular promoters in the absence of Tax suggests that they may play a role in basal HTLV-1 transcription. Within the context of the complete promoter, the contribution of individual TRE1s to basal transcription is unknown. Differences in the sequences of the TRE1s and the fact that these differences are conserved among different HTLV-1 isolates suggest that the TRE1s may be functionally distinct.

To specifically examine the role of individual TRE1s in HTLV-1 basal transcription, a C-to-T mutation was generated in the CRE core of each TRE1 in all combinations of one, two, or three TRE1 elements in the context of the complete LTR. Mutation of any single TRE1 reduced basal activity; however, the greatest reduction was observed when the middle TRE1 was mutated. Protein binding to the middle and TATA-distal TRE1s was dependent on the CRE core, while the preferred protein for binding to the proximal TRE1 was Sp1 and its binding was not affected by mutation of the CRE core. Further, Sp1 activated transcription from the proximal TRE1 in a dose-dependent manner. These studies demonstrate that Sp1 alone can activate HTLV-1 basal transcription through its interaction with the proximal TRE1 and that differential protein binding to the three TRE1s may mediate their functional differences in basal activity in vivo.

MATERIALS AND METHODS

Plasmids and oligonucleotides. All oligonucleotides were synthesized on a Beckman Oligo 1000 DNA synthesizer. The parent construct pU3RCAT has been described previously (38). Site-directed mutagenesis of pU3RCAT was performed with the Transformer mutagenesis kit (Clontech) by the method of Deng and Nickoloff (8). The oligonucleotides used to introduce the TRE1 mutations were 21P/11T (5′-CAGGCGTTGATGACAACCCCT-3′), 21M/11T (5′-TAGGCCCTGATGTGTCCCCCT-3′), and 21D/11T (5′-AAGGCTCTGATGTCTCCCCCC-3′). The position of the mutation is underlined in each sequence. The sequence of the selection oligonucleotide was 5′-ATTGTTGTTG ATATCTTGTTTATT-3′.

The entire promoter was sequenced from each of the resulting clones to ensure specificity of the mutations. Plasmid 11-2 contains a single copy of the proximal TRE1 and has been described previously (5). The Sp1 expression plasmids pPadhSp1-in (in frame) and pPadhSp1-out (out of frame) have been described previously (4). The competitors used in electrophoretic mobility shift assays (EMSAs) were Oct (5′-CTAGATGCTTTGCATTGCTTTGCAA-3′), CRE (5′-CTAGAGGCTGACGTCAGAG-3′), and Sp1 (5′-CTAGAGGGCGG GGGCGGGGGCGGA-3′).

Antibodies. Antibodies to Sp1 and AP2 were affinity-purified rabbit polyclonal immunoglobulin G purchased from Santa Cruz Biotechnology.

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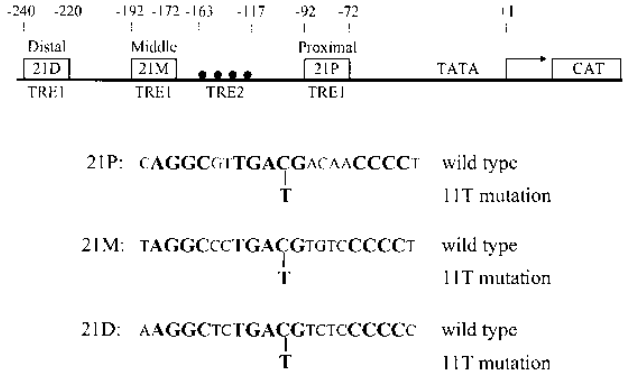

FIG. 1. Diagram of the HTLV-1 promoter. A linear representation of the HTLV-1 LTR promoter is shown at the top, and nucleotide positions are indicated. The three TRE1s (▭) and TRE2 (●●●●) are indicated. Nomenclature of the TRE1 sequences indicated in boxes: 21P, TATA-proximal TRE1; 21M, middle TRE1; 21D, TATA-distal TRE1. The specific sequence of each TRE1 is shown below. Sequences conserved among all three TRE1s are in boldface. The position of the 11T mutation is shown below each TRE1.

Cell culture and transfections. HeLa cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, and *Drosophila melanogaster* Schneider SL-2 cells were maintained in Schneider's *Drosophila* medium with 10% fetal calf serum. Cells were transfected 24 h after plating in 60-mm-diameter tissue culture dishes. Transfections were performed by the calcium phosphate method as described by Connor et al. (7). For HeLa cells, chloramphenicol acetyltransferase (CAT) reporter constructs (7 μg) were transfected along with 1 μg of a luciferase expression vector (pMSVLuc) as an internal control for transfection efficiency. pUC9 DNA and herring sperm DNA were added to keep the total DNA concentration constant at 20 μg. For Schneider cells, 1 μg of reporter plasmid and 1 μg of pMSVLuc were transfected alone or with increasing concentrations of the Sp1 expression vector (pPadhSp1-in). pPadhSp1-out and herring sperm DNA were added to keep the total DNA concentration constant at 20 μg. The transfections were harvested at 48 h posttransfection, using reporter lysis buffer (Promega) according to the manufacturer's protocol.

CAT assays. CAT assays were performed by the method of Seed and Sheen (36). For HeLa cells, each transfection was performed in duplicate and duplicate CAT assays were performed with extract from each plate. Reaction mixtures were incubated at 37°C overnight, and the amount of extract varied from 0.01 to 15 μl in order to keep the enzyme activity within the linear range. The results of four CAT assays (two CAT assays from each duplicate plate) were averaged, and the resulting counts were normalized for amount of extract used and luciferase activity. For Schneider cells, 50 μl of cell extract was used and the results are representative of three independent experiments.

Luciferase assay. Luciferase activity was measured in a Turner TD-20e luminometer. Luciferase assays were performed by adding either 25 μl of reporter lysis buffer (blank) or 25 μl of cell extract from the transfected cells to a 1.6-μl Turner Luminometer cuvette. Fifty microliters of luciferase assay substrate (Promega) was added to the sample, and luminescence of each sample was read relative to the blank.

EMSA. HeLa nuclear extract (2.0 μl, 6.2 μg/μl) (9) was mixed with 50,000 cpm of probe in each reaction. Probes were generated by annealing complementary single-stranded oligonucleotides, leaving 4-bp *Xba*I-compatible overhangs. The ends were filled in and labeled with Klenow enzyme (New England Biolabs) in the presence of [α-^{32}P]dCTP. EMSAs were performed with 6 μl of 5× gel shift buffer (50 mM HEPES [pH 7.9], 20% Ficoll, 250 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT]), 18 μl of buffer D (20 mM HEPES [pH 7.9], 100 mM KCl, 12.5 mM $MgCl_2$, 17% glycerol, 1 mM EDTA, 1 mM DTT), 0.5 μg of poly(dI-dC) (Boehringer Mannheim), 2 μl of HeLa nuclear extract, and 50,000 cpm of probe in a 30-μl total volume at room temperature for 20 min. In supershift experiments, antibody was added following the 20-min incubation and the reaction mixtures were incubated at room temperature for an additional hour. For reaction mixtures containing purified Sp1 protein, 30 μg of acetylated bovine serum albumin (New England Biolabs) was added, poly(dI-dC) was omitted, and Nonidet P-40 was added to 1% (vol/vol) final concentration. Complexes were separated on a 24-cm 5% acrylamide gel.

Purification of bacterially expressed CREB protein. CREB protein was expressed from plasmid pET156CREB341 in *Escherichia coli* BL21 and purified by a procedure modified from that of Hoeffler et al. (22). The cleared bacterial lysate was incubated for 15 min at 75°C. After purification, the protein was dialyzed against EMSA buffer D. The purity was estimated to be greater than 90% by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and Coomassie blue staining.

DNase I footprint analysis. Footprint probes were prepared by PCR amplification from a pU3RCAT template, using primers in which one of the primers had been labeled with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP. The 5′ primer was m21D described above and the 3′ primer, which anneals 68 bp downstream from the U3/R border, was 5′-CGCGACTCAACCGGCGTGGAT GGCGGCCTCAGG-3′.

PCR products were gel purified. Each probe (10,000 cpm) was mixed with increasing concentrations of purified Sp1 (Promega Corp.) and 25 μl of 2× binding buffer (50 mM Tris [pH 8], 0.1 M KCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 1 mM DTT) in a final volume of 50 μl for 10 min on ice. Each reaction was then supplemented with 50 μl of a solution containing 5 mM $CaCl_2$ and 10 mM $MgCl_2$ and incubated at room temperature for 1 min. Then 3 μl of RQ1 RNase-free DNase (0.05 U/μl) was added, and the reaction mixtures were incubated at room temperature for 100 s. The reactions were terminated by the addition of 90 μl of stop solution (0.2 M NaCl, 0.03 M EDTA, 1% SDS, 100 μg of yeast RNA per ml). The terminated reactions were extracted with phenol-chloroform-isoamyl alcohol, ethanol precipitated, and separated on a 6% acrylamide-urea gel. A sequencing ladder was prepared by using each primer on a pU3RCAT template and run in adjacent lanes for use as a size marker.

RESULTS

Effect of TRE1 mutations on LTR basal activity. To examine the contribution of individual TRE1s to HTLV-1 basal transcription, site-directed mutations were generated in the CRE core of each TRE1 within the HTLV-1 LTR reporter construct pU3RCAT. The mutations resulted in a C-to-T change in the position 11 of the 21-bp TRE1 sequence (Fig. 1). This mutation has previously been shown to render the distal TRE1 unresponsive to Tax in a model promoter (17). Seven mutants containing the possible combinations of single, double, and triple TRE1 mutations are shown schematically in Fig. 2.

Wild-type and mutant LTR constructs were transfected, and extracts from the transfected cells were tested for CAT enzyme activity. CAT activity has previously been shown to accurately represent transcriptional activity of pU3RCAT (5). Combined mutation of all three TRE1 elements (21PMD/11T) reduced transcription 38-fold compared to the wild-type level (106 CAT units versus 4,028 CAT units [Fig. 2]). Mutation of one or two TRE1s resulted in intermediate levels of transcription reduction; however, the degree of reduction was dependent on the

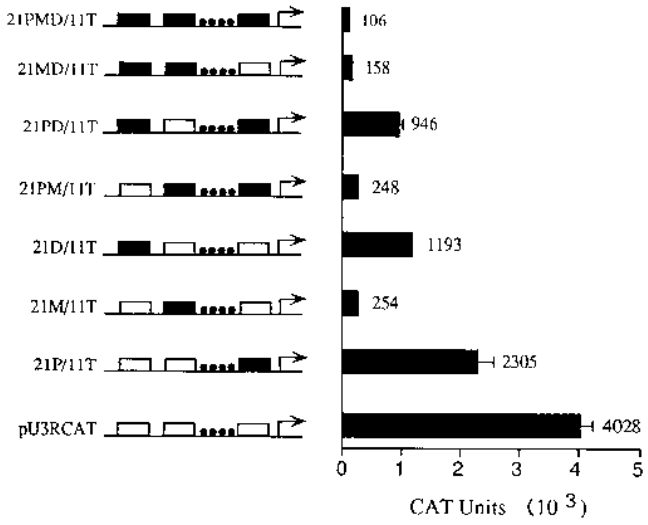

FIG. 2. Basal transcription of the HTLV-1 promoter mutants. Diagrams of the eight CAT reporter constructs are shown on the left. Black boxes indicate that the TRE1 contains the 11T mutation. The wild-type HTLV-1 LTR reporter (pU3RCAT) or the indicated mutant was transfected into HeLa cells. CAT units were determined by normalizing the results obtained from the CAT assay to luciferase activity. Error bars represent the range of values obtained from the four assays. Where no error bars are shown, the variation was too small to be visible on the graph.

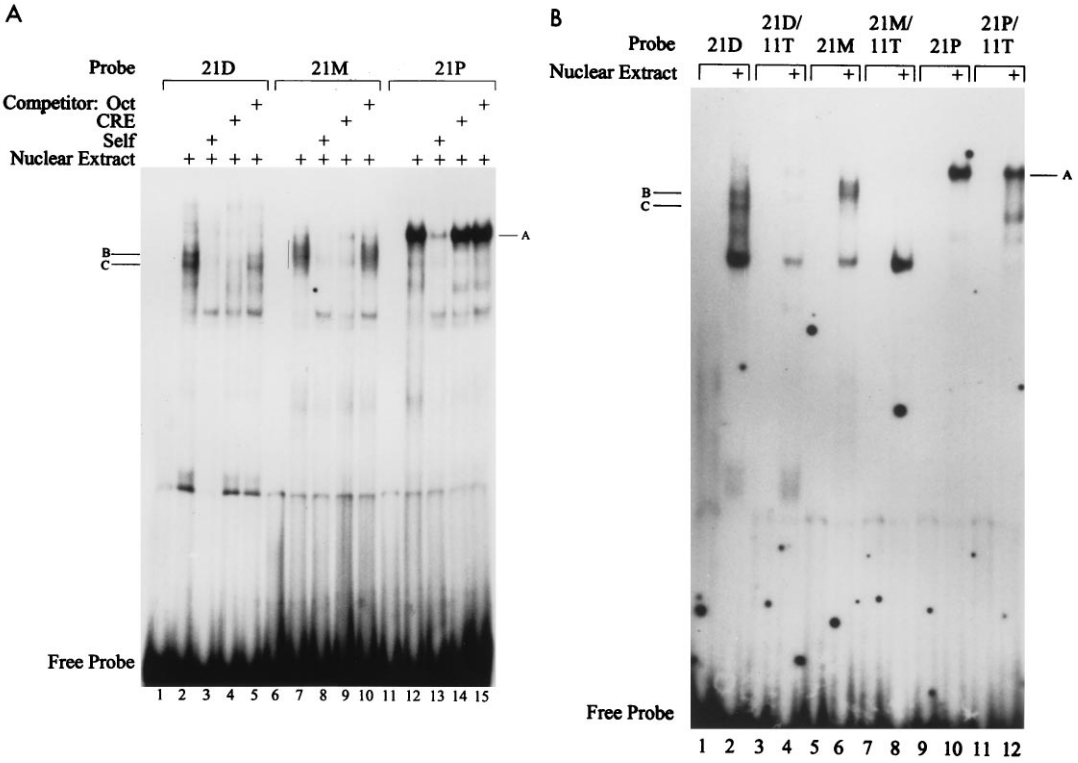

FIG. 3. The three TRE1 sequences bind different proteins in vitro. (A) Protein complexes formed on each of the three TRE1s were analyzed by incubating probe alone (lanes 1, 6, and 11) or probe plus nuclear extract (lanes 2 to 5, 7 to 10, and 12 to 15). Probe 21D was used in lanes 1 to 5, 21M was used in lanes 6 to 10, and 21P was used in lanes 11 to 15. The presence of a 40-fold molar excess of unlabeled competitors is indicated above the appropriate lanes. The self competitors are 21D on the 21D probe, 21M on the 21M probe, and 21P on the 21P probe. A diffuse complex observed on the 21M probe is indicated by a line beside lane 7. Positions of the complexes are shown on the left (B and C) and right (A). (B) Effect of the 11T mutation on binding to the three TRE1 probes. Each of the three wild-type probes is shown adjacent to the respective 11T mutant. The identity of each probe is indicated above. The odd-numbered lanes show probe migration in the absence of nuclear extract, while the even-numbered lanes show complexes formed in the presence of nuclear extract. Locations of specific complexes formed on the wild-type probes are indicated on the left (B and C) and right (A).

specific TRE1(s) mutated. Single mutation of the middle TRE1 (21M/11T, 254 CAT units) had a greater effect on basal transcription (16-fold reduction) than mutation of the TATA-proximal (21P/11T, 2,305 CAT units, 1.7-fold reduction) or TATA-distal (21D/11T, 1,193 CAT units, 3.4-fold reduction) element. Also, when the proximal and distal TRE1s were both mutated (21PD/11T, 946 CAT units), leaving a wild-type middle TRE1, four to six times more CAT activity was observed than with either of the other double mutants (21PM/11T, 248 CAT units; 21MD/11T, 158 CAT units), again suggesting that the middle TRE1 contributes significantly to basal activity.

The three TRE1s bind different protein complexes. Since the TRE1s contain variations in nucleotide sequence and since mutations in the TRE1s had different effects on basal transcription, it was possible that different proteins bind to these elements. Several previous studies have demonstrated variations in the complement of protein complexes formed on each TRE1 within the HTLV-1 LTR; however, the specificity of these complexes and their dependence on the CRE core have been carefully examined only on the distal TRE1 (43, 44). Thus, the complexes formed on each wild-type TRE1 were examined by EMSA using oligonucleotide probes (Fig. 3A). With the distal TRE1 (21D) as a probe (lanes 1 to 5), two major complexes (B and C) as well as several minor complexes were observed. Complexes B and C were specific for the probe since they were competed by a 40-fold molar excess of unlabeled probe (self) but not by a 40-fold molar excess of the unrelated octamer binding site oligonucleotide (Oct). These complexes appeared to result from binding to the CRE core of TRE1 since they were competed by a 40-fold molar excess of an oligonucleotide containing the rat somatostatin CRE.

When the middle TRE1 (21M) was used as a probe (Fig. 3A, lanes 6 to 10), a broad complex that did not resolve into discrete bands was observed (lane 7, bar). This complex was specific for 21M and the CRE core since unlabeled 21M and CRE oligonucleotides competed for its formation and the Oct oligonucleotide did not. This broad complex migrated more slowly than complexes B and C observed with the distal TRE1 probe, suggesting that the proteins that comprise this broad complex are not identical to those in complexes B and C formed on the distal TRE1. Binding of CREB and ATF family members to the TRE1s has been previously described (11) and confirmed by us (data not shown).

When the proximal TRE1 (21P) was used as a probe (Fig. 3A, lanes 11 to 15), a single major complex, A, was resolved. This complex was specific since it was competed by unlabeled probe, but unlike the major complexes formed on 21D and 21M, it was not competed by the CRE or Oct oligonucleotide. Previous studies have shown that CRE-dependent complexes can form on the proximal TRE1 in vitro, using different EMSA conditions and nuclear extracts from different cell types (6, 32, 43). We have also observed that members of the CREB/ATF family bind to the proximal TRE1 with lower affinity than to the middle or distal TRE1 (data not shown). However, with nuclear extract, no CRE-specific complexes were observed on the 21P probe. Thus, banding patterns differed for all three

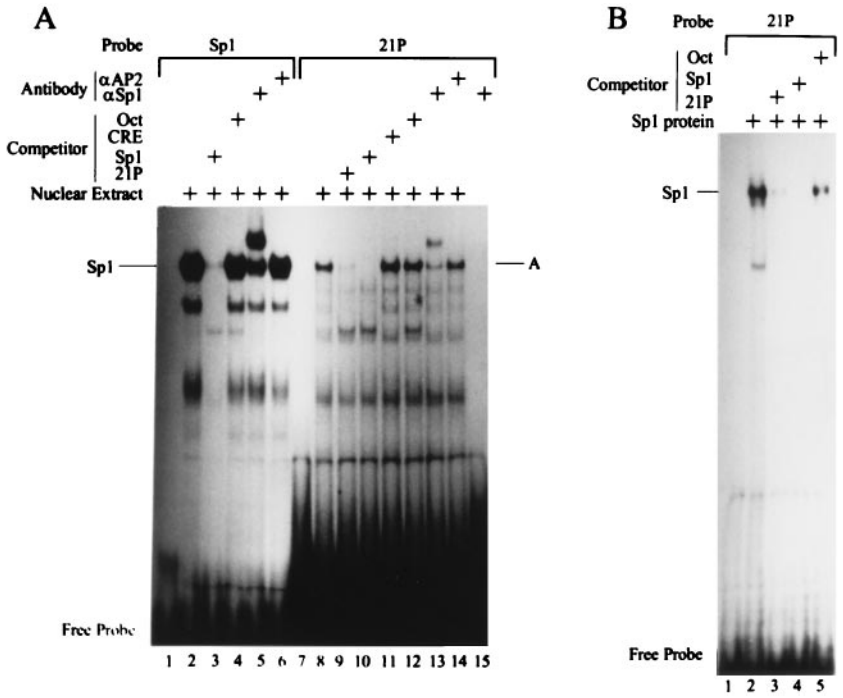

FIG. 4. Sp1 binds to the proximal TRE1. (A) The identity of the major complex on the 21P probe (lanes 7 to 15) was determined by comparison with a probe containing three tandem, consensus Sp1 binding sites (lanes 1 to 6). The migration of the probes in the absence of nuclear extract is shown in lanes 1 and 7. Competitor oligonucleotides were used in 40-fold excess as indicated above the appropriate lanes. Polyclonal antibodies to Sp1 (αSp1; lanes 5, 13, and 15) and AP2 (αAP2; lanes 6 and 14) were included in the indicated reactions. (B) The ability of Sp1 protein to bind the proximal TRE1 was demonstrated by incubation of the 21P probe (lane 1) with purified Sp1 (lanes 2 to 5). Unlabeled oligonucleotides were added to the reactions in 40-fold molar excess as indicated (lanes 3 to 5) to demonstrate binding specificity.

probes. These results are consistent with the observation that extracts from lymphoid and neural cells form different protein complexes on each of the TRE1s (43). Our DNA-protein binding results extend this previous study by demonstrating the specificity of the complexes. Further, our results suggest that the differences in basal activity observed with the LTR mutants in vivo could be due to differences in the protein complexes that bind the TRE1s in vivo.

Effect of 11T mutations on protein binding. Since the 11T mutation in individual TRE1s had different effects on basal transcription in vivo, it was important to determine the effects of the 11T mutation on protein binding to each TRE1. Therefore, EMSAs were performed with probes containing the same C-to-T mutation that was characterized for in vivo activity. Wild-type and mutant probes were run in adjacent pairs of lanes in order to compare complexes (Fig. 3B). The two specific complexes (B and C) seen on the 21D probe failed to form on the 21D/11T mutant probe (compare lanes 2 and 4). The series of specific complexes formed on the 21M probe also failed to form on its corresponding mutant, 21M/11T (compare lanes 6 and 8). The fact that specific binding was completely abrogated on the middle TRE1 mutant probe (21M/11T) and that this mutation had the greatest effect on basal transcription in vivo suggests that these particular complexes play an important role in HTLV-1 basal transcription. The 21P/11T mutant probe retained the ability to form complex A observed with the wild-type 21P probe (compare lanes 10 and 12), and the complex formed on the mutant probe was specific (data not shown). An additional complex observed on the 21P/11T probe migrated below complex A. Since the proximal mutation did not affect protein binding and retained near wild-type transcriptional activity, the possibility remains that this element contributes to basal control of the HTLV-1 promoter. Taken together, these results demonstrate that the 11T mutation did not affect specific protein complex formation on 21P, but the 11T mutants of 21D and 21M were unable to form the complexes of their wild-type counterparts.

Sp1 binds the proximal TRE1. The major complex (complex A) observed on the 21P probe was shown to contain Sp1 by a series of EMSAs. Complexes formed on a prototypic Sp1 binding site probe and on the 21P probe migrated to the same position (Fig. 4A; compare lanes 2 and 8). On both probes, complex A was competed by unlabeled Sp1 oligonucleotide (lanes 3 and 10) but not by unlabeled Oct oligonucleotide (lanes 4 and 11). Similar Sp1-specific complexes were observed when extracts from the human T-lymphocyte cell line CEM were used (data not shown). As observed in Fig. 3, complex A formed on the 21P probe was not competed by a CRE oligonucleotide (Fig. 4A, lane 12). When used as competitors, the wild-type middle and distal TRE1s did not affect the binding of Sp1 to the proximal TRE1 (data not shown). Polyclonal antisera against Sp1 caused a supershift on both the Sp1 and 21P probes (lanes 5 and 13). An antibody directed against the cellular transcription factor AP2 did not cause a supershift on either probe (lanes 6 and 14), and the Sp1 antibody did not bind the 21P probe in the absence of nuclear extract (lane 15). These results demonstrate that Sp1 protein is contained in complex A formed on the proximal TRE1 probe.

To determine whether Sp1 can bind the proximal TRE1 in the absence of other proteins, EMSA was performed with purified human Sp1 and the 21P probe (Fig. 4B). The purified Sp1 protein formed a complex with the 21P probe (lane 2). Unlabeled 21P and Sp1 oligonucleotides competed for formation of the complex whereas unlabeled Oct oligonucleotide did

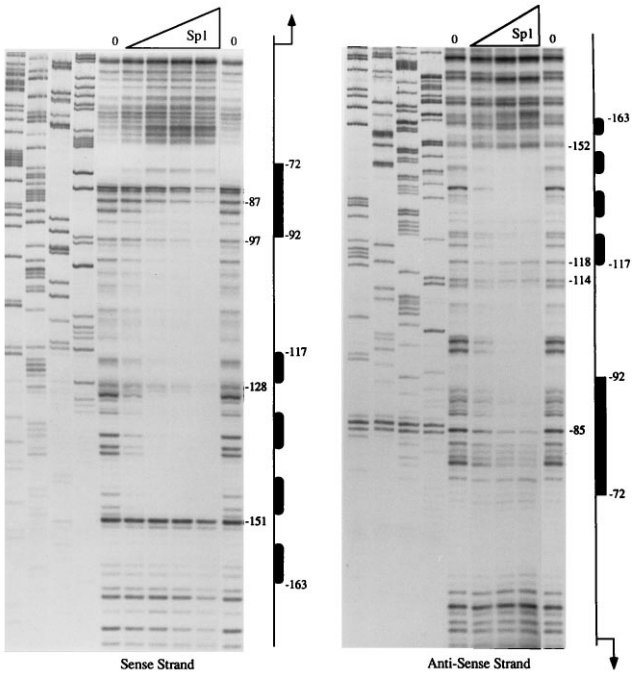

FIG. 5. Footprint analysis of Sp1 binding to the HTLV-1 LTR. Binding of Sp1 protein to the sense and antisense strands of the LTR probe is shown. Increasing amounts of Sp1 protein were incubated with the sense probe (0.25, 0.5, 1.0, or 2.0 footprint units) or the antisense probe (0.5, 1.0, or 2.0 footprint units). A DNA sequence of each probe is shown on the left. A schematic diagram of the LTR is positioned next to each strand, with the nucleotide position of LTR elements indicated on the right and footprint boundaries indicated on the left of each diagram.

not, demonstrating binding specificity (lanes 3 to 5). Also, complex A, which forms on 21P in the presence of nuclear extract, comigrates with the complex observed on 21P with purified Sp1 (data not shown). Together, these results demonstrate independent and specific binding of Sp1 to the proximal TRE1 to form complex A observed with nuclear extract.

In addition to TRE1, the HTLV-1 promoter contains another Tax-responsive element, TRE2, which binds multiple cellular transcription factors, including c-Ets-1, NF-κB, p36, THP-1, and THP-2 (14, 18, 28, 31, 42). Sp1 binding to TRE2 has also been demonstrated in a DNase I footprint assay (32). We used DNase I footprint analysis to determine the position of Sp1 binding within the vicinity of the proximal TRE1. Increasing amounts of purified Sp1 protein were incubated with singly end-labeled probes representing the sense or antisense strand of the HTLV-1 LTR. Protected regions were localized by alignment with DNA sequence markers. Sp1 binding, which covered portions of TRE2 and the proximal TRE1, was detected on both the sense and antisense strands (Fig. 5) with approximately the same boundaries. The upstream limit of protection was nucleotide −151 on the sense strand and nucleotide −152 on the antisense strand. The downstream limit of protection was −87 on the sense strand and −85 on the antisense strand. This large footprint was interrupted at positions −97 and −128 on the sense strand and −114 and −118 on the antisense strand. The footprint covered the middle and 5′ ends of the proximal TRE1. Particularly evident on the sense strand, the TRE2 element was fully loaded with Sp1 at lower protein concentrations (0.5 footprint unit) than the proximal TRE1 (2.0 footprint units). These results suggest that Sp1 binding may be nucleated on TRE2, and cooperative interactions may contribute to Sp1 binding to the proximal TRE1. While this type of interaction may occur on the intact LTR, the proximal element is capable of binding Sp1 independently of other LTR elements, based on the EMSA results presented in Fig. 3 and 4.

Previous studies have demonstrated that the cellular transcription factor CREB can bind to the proximal TRE1 (6, 32, 43); thus, we were interested in determining whether CREB and Sp1 can occupy the proximal TRE1 at the same time. In an assay using purified Sp1 protein, the proximal TRE1 probe formed the expected complex with Sp1 protein alone (Fig. 6, lane 2). Upon addition of increasing concentrations of purified CREB, the Sp1 band disappeared and a CREB-specific complex was formed. At approximately equimolar concentrations of Sp1 and homodimeric CREB, both complexes were observed on separate probe molecules (lane 5), suggesting that CREB and Sp1 have similar affinities for the proximal TRE1. These results demonstrate that the bindings of CREB and Sp1 to the proximal TRE1 are mutually exclusive. Since Sp1 is one of the most prevalent transcription factors, it will usually be the preferred proximal TRE1-binding protein; however, factors which affect either the affinity or the availability of CREB or Sp1 in the nucleus may alter the identity of the proximal TRE1-binding protein.

Transcriptional activation of the HTLV-1 LTR by Sp1. Since these studies revealed Sp1 binding to the proximal TRE1, it was important to determine whether Sp1 could functionally activate basal transcription from the LTR. The *Drosophila* Schneider SL-2 cell line, which is devoid of functional Sp1, was transfected with increasing concentrations of an Sp1 expression construct and a reporter either containing the complete wild-type U3/R region of the HTLV-1 LTR, pU3RCAT, or containing a single copy of the proximal TRE1, 11-2. Sp1 activated both reporters in a dose-dependent manner, with maximum activations of 90- and 160-fold on pU3RCAT and 11-2, respectively (Fig. 7). It should be noted that the absolute activity of these reporters in the absence of Sp1 was quite low,

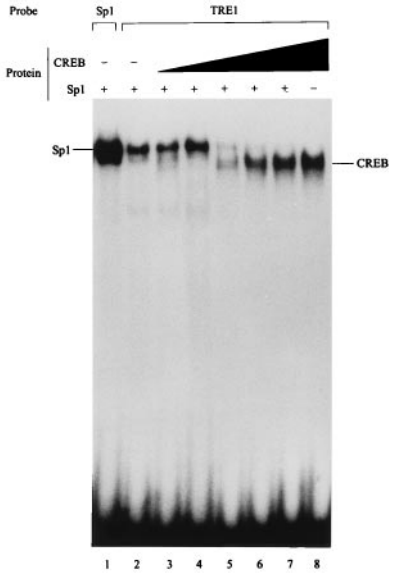

FIG. 6. Competitive binding of Sp1 and CREB to the proximal TRE1. Sp1 (lane 1) and TRE1 (lanes 2 to 8) probes were incubated with 25 ng of purified Sp1 protein (lanes 1 to 7). Increasing concentrations of purified CREB were included in lanes 3 to 8 (5, 15, 25, 50, 100, and 665 ng, respectively). Positions of Sp1 and CREB complexes are shown on the left and right, respectively.

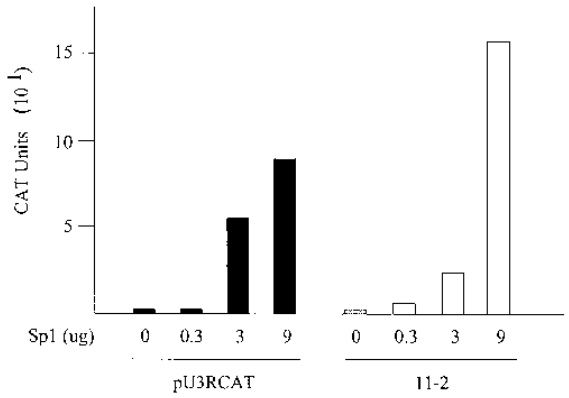

FIG. 7. Sp1 activation of HTLV-1 basal transcription. The *Drosophila* Schneider SL-2 cell line was transfected with 1 μg of the reporter construct pU3RCAT (■) or 11-2 (▨). The reporters were transfected either alone or in combination with 0.3, 3, or 9 μg of the Sp1 expression vector, pPadhSp1-in. The CAT activity was normalized based on luciferase activity of each extract. CAT units were calculated by dividing the normalized CAT activity of transfections containing Sp1 by the normalized CAT activity of the same reporter in the absence of Sp1.

which is reflected in the relatively high fold activation observed upon addition of Sp1 and further implicates Sp1 as an important regulator of HTLV-1 basal transcription. While Sp1 activation of pU3RCAT could utilize Sp1 binding sites in either TRE2 or the proximal TRE1, the 11-2 reporter does not contain TRE2, suggesting that Sp1 activation of this reporter occurs through the proximal TRE1. These results suggest that Sp1 contributes to basal transcription regulation of the HTLV-1 LTR and that the proximal TRE1 is independently able to mediate transcriptional activation by Sp1.

DISCUSSION

The DNA elements and cellular proteins involved in basal transcription regulation of the HTLV-1 LTR have not been well characterized. Rather, analysis of transcription control elements has focused primarily on Tax responsiveness. These studies have been performed in model systems using multiple TRE1s to mediate Tax transactivation. While some mutagenesis of the three TRE1 sequences in the wild-type HTLV-1 LTR has been performed (29, 37), no systematic analysis of the role of each individual TRE1 in HTLV-1 basal transcription has been reported. Thus, a specific C-to-T mutation was generated at position 11 in one, two, or all three TRE1s within the context of the complete 5′ LTR. This mutation had previously been shown to abolish Tax responsiveness of the distal TRE1 (17, 29). Analysis of the mutants revealed that the middle TRE1 had the greatest effect on basal transcription, followed in order by the distal and proximal TRE1s. Double TRE1 mutations reduced promoter activity further. However, when the middle TRE1 was wild type, greater basal activity was observed. The triple mutant showed the greatest reduction in activity (38-fold).

To determine the basis for the differential effects of the TRE1 mutations on basal LTR activity in vivo, the binding of proteins to these elements was examined in vitro. Different complexes bound the three TRE1s in vitro, correlating with the differential effects of each TRE1 mutation on basal activity. The major complexes formed on the distal TRE1 were CRE dependent; however, several minor complexes that were not CRE dependent were formed. Mutation of the CRE core within the distal TRE1 abolished all of these complexes and resulted in a 3.4-fold reduction in basal transcription.

The middle TRE1 bound a heterogeneous group of complexes which were all specific for the CRE core and likely represented a variety of heterodimers formed from the ATF/CREB family of transcription factors. This possibility is supported by the findings that purified CREB and ATF proteins bound a middle TRE1 probe and migrated to a position similar to that of the broad complex formed in the presence of nuclear extract. Further, both CREB and ATF antibodies could supershift the complex formed on the middle TRE1 probe (data not shown). The subtle variation in sequence surrounding the CRE core may dictate the specific complement of ATF/CREB heterodimers that can bind to a given TRE1. Since it has been shown that CREB homodimers are more responsive to cyclic AMP than heterodimers of ATF-1 and CREB (10), it is likely that the particular complement of DNA-protein complexes which form on the middle TRE1 confers to it a greater role in basal activity. It is also possible that its position within the LTR allows the middle TRE1 to make the greatest contribution to basal activity.

The proximal TRE1 binds Sp1, and mutation of its CRE core does not appreciably affect Sp1 binding. The ability of the 21P/11T mutant to retain Sp1 binding may explain its transcriptional activity (58% of the activity of the wild-type LTR). The fact that some reduction in basal activity was observed with 21P/11T suggests that the mutation may have a subtle effect on Sp1 binding. This possibility is supported by the observation that the Sp1 footprint covered the CRE core of the proximal TRE1.

To directly determine whether binding of Sp1 to the proximal TRE1 was biologically significant, its ability to activate transcription from the complete LTR or from the proximal TRE1 alone was examined. In addition to the proximal TRE1, the complete LTR contains a TRE2 sequence which has previously been shown to bind Sp1 (32). Therefore, the dose-dependent activation of the complete LTR by Sp1 could have been due to the TRE2 site, the proximal TRE1 site, an as yet unidentified site, or a combination of sites. The ability of the 11-2 reporter, which contains only a proximal TRE1, to respond to Sp1 in a dose-dependent manner demonstrates that this element is independently able to mediate Sp1 activation. Since cooperative binding of Sp1 to multiple DNA elements has been shown, it is possible that in the context of the complete LTR, TRE2 and the proximal TRE1 cooperate in response to Sp1.

The precise sequence requirements for Sp1 binding to the proximal TRE1 have not yet been determined; however, footprint analysis suggests that the Sp1 binding site is localized in the 5′ end of the proximal TRE1. The sequence AGGCGT in the 5′ end of the proximal TRE1 differs from the consensus Sp1 binding site, GGGCGG, in the two outside positions and is a likely candidate for the Sp1 binding site. This sequence in the proximal TRE1 is identical to the upstream Sp1 binding site (III) in the human immunodeficiency virus type 1 LTR (24). It should be noted that Sp1 binding was not detected on either the middle or distal TRE1 and that these elements differ from the proposed proximal Sp1 binding site at position 5, which is conserved between the proximal TRE1 and the consensus Sp1 binding site. The presence of functional Sp1 binding sites which differ, in some cases markedly, from the consensus have been identified in other viruses, including the E2 promoter of bovine papillomavirus, the human immunodeficiency virus type 1 LTR, and the cytomegalovirus IE-1/2 promoter (24, 26, 39).

Sp1 has previously been shown to play an important role in

basal transcription of other promoters (25), and our studies suggest that it plays a similar role in HTLV-1 basal transcription. However, to attain high levels of activated transcription, the viral promoter must be responsive to the Tax protein. Other investigators have demonstrated that in the presence of Tax, the DNA binding affinity of the bZIP family of transcription factors, which includes CREB, is increased (1, 2, 11, 44). We have demonstrated that the bindings of Sp1 and CREB to the proximal TRE1 were mutually exclusive. This type of competitive binding has also been observed for Sp1 and CREB binding to the cytomegalovirus IE-1/2 promoter (26). Using this information, we propose the following model for use of the proximal TRE1 in basal and Tax-activated transcription. Sp1 is the preferred proximal TRE1-binding protein in the absence of Tax and helps to regulate basal HTLV-1 transcription along with proteins that bind the middle TRE1. Once the doubly spliced Tax message is transcribed and translated, the presence of Tax in the nucleus can increase the affinity of CREB for the proximal TRE1. Since the bindings of Sp1 and CREB are mutually exclusive, CREB will replace Sp1 as the preferred proximal TRE1-binding protein, providing an additional position for loading of Tax onto the promoter. Presumably, increased loading of Tax onto the LTR is advantageous for transcriptional activation. Using this arrangement of transcription factor binding sites, HTLV-1 appears to have optimized the use of viral genetic information.

In summary, the HTLV-1 promoter contains a complex series of overlapping binding sites for numerous transcription factors. It is likely that a number of these elements and binding proteins function coordinately to regulate basal transcription. Because of the clinical latency associated with HTLV-1 infection, basal transcription may be an important determinant of disease progression. The results presented in this study provide new in vivo insight into the role of individual TRE1s in HTLV-1 promoter function. Specifically, we cannot consider the three TRE1 elements to have identical protein binding capacities and identical functions in transcription control. Further, Sp1 has been identified as a potentially important regulator of basal HTLV-1 transcription.

ACKNOWLEDGMENTS

We thank Richard Goodman (Oregon Health Sciences University) for supplying plasmid pET156CREB341, Robert Tjian (University of California, Berkeley) for supplying plasmids pPadhSp1-in and pPadhSp1-out, Mitzi Kuroda and Ron Richman (Baylor College of Medicine) for Schneider SL-2 cells, Mark Goff and Andrew Rice for critical evaluation of the manuscript, and Kim Payne for excellent technical assistance.

M.K.B. was supported in part by NIH training grant T32AI07483. L.M.C. was supported in part by NIH training grant T32CA09197. This work was supported by a National Institutes of Health research grant awarded to S.J.M.

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